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Effects of Water Pollutants and Other Chemicals on Fish Acetylcholinesterase (In vitro)

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Acetylcholinesterase (AChE) preparations from the muscle of the fathead minnow (Pimephales promelas Rafinesque) were treated (in vitro) with 74 chemicals of various classes, many of which are environmental contaminants, to determine their effect upon enzyme activity. A highly inhibitory effect was found with several carbamates, one organo-oxy-phosphate, the arsenite ion, and certain heavy metal cations. Intermediate inhibition was found with the arsenate ion, other metal cations, organometals, certain neuroactive agents, organophosphates, and one organochloride pesticide. Several organochloride pesticides, alkaline and alkaline-earth cations, a variety of anions, several drugs, and other chemicals had little inhibitory effect at the highest concentration used (0.01 M). The effect of chemicals in mixtures of known composition was approximately additive. None of the chemicals tested caused detectable enzyme activation.

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ENVIRONMENTAL RESEARCH 21, 327–335 (1980)

Effects of Water Pollutants and Other Chemicals on Fish Acetylcholinesterase (*in Vitro*)

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Acetylcholinesterase (AChE) preparations from the muscle of the fathead minnow (*Pimephales promelas* Rafinesque) were treated (*in vitro*) with 74 chemicals of various classes, many of which are environmental contaminants, to determine their effect upon enzyme activity. A highly inhibitory effect was found with several carbamates, one organo-oxy-phosphate, the arsenite ion, and certain heavy metal cations. Intermediate inhibition was found with the arsenate ion, other metal cations, organometals, certain neuroactive agents, organophosphates, and one organochloride pesticide. Several organochloride pesticides, alkaline and alkaline–earth cations, a variety of anions, several drugs, and other chemicals had little inhibitory effect at the highest concentration used (0.01 M). The effect of chemicals in mixtures of known composition was approximately additive. None of the chemicals tested caused detectable enzyme activation.

INTRODUCTION

Cholinesterase research began many years ago with the determination of the active agents in the plant *Physostigma venenosum*, which led to an explanation for their physiological effects upon humans (Silver, 1974). The alkaloid eserine (physostigmine) in the bean of this plant has a very specific affinity for acetylcholinesterase (AChE) and induces its physiological effect through inhibition of this enzyme. The literature on the biological, physical, and pharmaceutical chemistry of cholinesterases is considerable and includes data on activators and inhibitors (Wilson, 1963; Karczmar, 1970; Heller and Hanahan, 1971; Fest and Schmidt, 1973; Ecobichon and Comeau, 1973; Goldberg and Hanin, 1976). Most of the work on specific anticholinesterase agents and other reaction-rate-influencing chemicals (*in vivo* and *in vitro* studies) has been concerned with carbamates and amine-carbamates (oxy- and thio-), alkylphosphates (oxy- and thio-), selenophosphates, fluorophosphates, and organosulfonates (Hayama and Kuwabara, 1962; Davis and Malaney, 1967; O Brien, 1967; Voss, 1969; Karczmar, 1970; Su *et al.*, 1971; Corbett, 1974; Mukherjee and Bhattacharya, 1974; Thirugnanam and Forgash, 1975; Coppage, 1977). Many chemicals in these classes are used as pesticides or other types of biocides, and many have thus become environmental contaminants. Chemicals other than carbamates and organophosphates that are environmental contaminants or that have ecological significance and have been found to affect the activity of cholinesterases are compounds of copper, mercury, lead, iron, and nickel (Abou-Donia and Menzel, 1967), and alkyl lead (Galzigna *et al.*, 1969; Heywood *et al.*, 1978). Chemicals of other structure classes have also been investigated (Karczmar, 1970; Mukherjee and Bhattacharya, 1974).

Because of the reported sensitivity of AChE to carbamate and organophosphate pesticides, the enzyme has been used as a biochemical indicator of pollution by

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these agents (Williams and Sova, 1966; Davis and Malaney, 1967; Gage, 1967; Nicholson, 1967; Coppage and Braidech, 1976). However, the molecular structure and chemical reactivity for many classes of chemicals has not been closely correlated with their effect upon AChE, and it is difficult at this stage of knowledge to predict biological effects based only on structure.

In the current *in vitro* study the effect on the activity of AChE from the muscle of the fathead minnow (*Pimephales promelas* Rafinesque) was determined for 74 chemicals. The substances tested were chosen from many different chemical classes and included important water pollutants, neurochemical drugs, and certain neurobiological agents. In addition, mixtures of chemicals were tested to determine their effect on AChE activity. The combined effects of chemicals have particular relevance to environmental pollution, because polluted air, land, and water usually contain a variety of chemicals. Individually, these agents below certain concentrations may not stimulate an identifiable toxic response in animals, but when combined the sum of all effects may be very significant.

MATERIALS AND METHODS

Mature fish of both sexes were obtained from stock supplies cultured at the Environmental Research Laboratory in Duluth, Minnesota. Fifty fish were decapitated and deviscerated, and the remaining tissue, which weighed about 50 g, was homogenized at 1°C in 200 ml of Tris buffer (0.05 M; pH 7.0), first with a blender at slow speed and then a tissue grinder. The debris was removed by centrifugation (1000*g* for 15 min at 1°C), and the supernatant was portioned into small vials and stored at −20°C until analysis.

Enzyme activity and protein concentration were determined simultaneously following, in general, published methods.[1] A diagram of the semiautomated layout is shown in Fig. 1. Bovine serum albumin (from 0.1 to 1 g%) was used as the protein standard, and acetyl thiocholine iodide (20 mM) was used as the enzyme substrate with glutathione (from 0.1 to 5 mM) as the reference standard. The reaction preincubation mixture was set up so that 200 μl of the enzyme (fish tissue) preparation was added to 100 μl of the test chemical in water or 50% aqueous acetone (water-insoluble reagents) for 15 min. The concentration of the color-inducing reagent, 5,5-dithio-bis-2-nitrobenzoic acid, was 0.2 mM. The concentration range studied for most of the test chemicals was from 1.0×10^{-2} M to that minimum concentration where no change in activity was noted. Reagent blanks were determined with each series of test reagents, and the pH of the autoanalyzer reaction sampler was continuously monitored with a flow-through electrode, which was coupled to a meter with a stripchart recorder. All experiments were done at least three times. Protein was determined as grams per 100 ml of preparation, and AChE as specific activity = μM product per milligram of protein per minute. The variability of the analyses was about ±5%.

The 74 organic and inorganic chemicals selected for study were representative of some of the more important chemical classes of water pollutants, but included

[1] Technicon Instrument Co., Tarrytown, N.Y. 10591. Methods: total protein, *n*-86, AChE, 354-75P-A (modified).

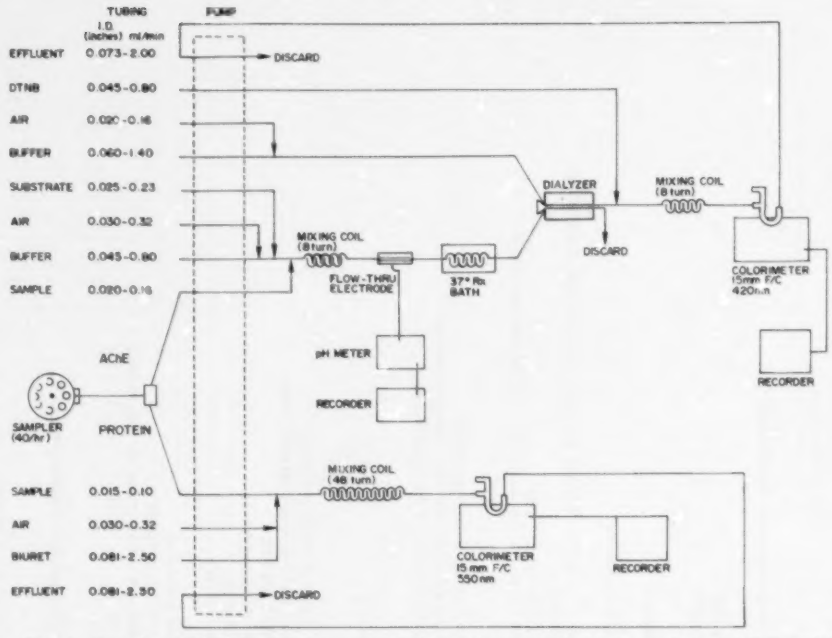

FIG. 1. Reaction module for the semiautomated quantitative analysis of protein and AChE activity in fish preparations.

biochemicals, pharmaceuticals, and other reagents (Table 1). In addition to the individual tests with these chemicals, three mixtures were tested for their effect on AChE activity: (1) 10 inorganic chemicals ($HAuCl_4 \cdot 3H_2O$, K_2PdCl_6, $PtCl_4$, $CdCl_2 \cdot 2½H_2O$, $CuCl_2 \cdot 2H_2O$, $HgCl_2$, $AlCl_3$, $CrCl_3 \cdot 6H_2O$, $SnCl_2 \cdot 2H_2O$, and $ZnCl_2$); (2) 10 pesticides (malathion, DDT, diazinon, captan, lindane, eptam, IPC, dieldrin, aldrin, and thiram); and (3) all components of mixtures (1) and (2).

The amount of each component in a preparation was based upon that concentration which alone constituted a threshold (effect–no effect) value. Dilutions were made of these preparations, and enzyme analyses were carried out as with single toxicant solutions.

RESULTS AND DISCUSSION

Of the 74 chemicals analyzed, eserine was most reactive with AChE (Table 2), which demonstrates again that this di-tertiary amine-carbamate has a supreme inhibiting effect on this enzyme. The carbamate neostigmine was slightly less reactive, and the carbamate pesticide carbaryl was about 31 times less reactive than eserine. The carbamate IPC was about 5000 times less inhibitive than eserine. The organophosphate malaoxon was much more inhibiting than its sulfurated analogue malathion, which agrees with the work of Karczmar (1970), who reported that many organothiophosphate pesticides were more inhibiting after becoming

TABLE 1 ORGANIC AND INORGANIC CHEMICALS TESTED FOR THEIR EFFECT ON ACETYLCHOLINESTERASE (AChE) ACTIVITY

Common name	Chemical description
A. Thiomides	
1. Captan	*N*-(Trichloromethylthio)-4-cyclohexene-1,2-dicarbioximide
B. Thiocarbamates	
2. Thiram	Tetramethylthiuram disulfide
3. Eptam	*S*-Ethyl dipropylthiocarbamate
C. Carbamates	
4. IPC	Isopropyl-*N*-phenyl carbamate
5. Eserine (SO_4)	Physostigmine
6. Carbaryl	Sevin; 1-Naphthyl-*N*-methyl carbamate
D. Halogenated hydrocarbons	
7. Aldrin	1,2,3,4,10,10-Hexachloro-1,4,4*a*,5,8,8*a*-hexahydro-endo-exo-1,4:5,8-dimethanonaphthalene
8. DDT	Dichlorodiphenyltrichloro-ethane
9. Lindane	1,2,3,4,5,6-Hexachlorocyclohexane (γ isomer)
10. Dieldrin	1,2,3,4,10,10-Hexachloro-6,7-epoxy-1,4,4*a*,5,6,7,8,8*a*-octahydro-endo-exo-1,4:5,8-dimethanonaphthalene
E. Organothiophosphate	
11. Malathion	*S*-1,2-Dicarbethoxyethyl)-*O*-*O*-dimethyldithiophosphate
12. Diazinon	*O*-*O*-Diethyl-*O*-(2-isopropyl-4-methyl-6-pyrimidyl) phosphorothionate
13. Malaoxon	*O*-*O*-Dimethyl-*S*-(1,2-dicarboethoxyethyl) phosphotothionate
F. Organometals	
14. Methylmercuric chloride	
15. PCMB	4-Chloromercuribenzoic acid
16. Phenylmercuric chloride	
G. Neuroactive agents	
17. DL-Noradrenaline (HCl)	DL-Amino-1-hydroxy-1-(3,4-dihydroxyphenyl) ethane
18. L-Adrenaline	L-2-Methyl-amino-1-hydroxy-1-(3,4-dihydroxyphenyl) ethane
19. Bufotenin	*N*,*N*-Dimethyl-5-hydroxy tryptamine
20. Serotonin	(creatinine·SO_4)-5-Hydroxy tryptamine
21. PAM	Pyridine-2-aldoxime methiodide
22. Nicotine	1-Methyl-2-(3-pyridyl)-pyrrolidine
23. Caffeine	1,3,7-Trimethyl xanthine
24. Phenacetin	*N*(4-Ethoxyphenyl) acetic acid amide
25. Atropine ($SO_4 \cdot 2H_2O$)	1-*a*-H,5*a*H-Tropan-3-*a*-01(±)-tropate ester
26. DL-Muscarine (Cl)	2-Trimethylammoniummethyl-4-hydroxy-5-methyltetrahydrofuran
27. Chloral hydrate	Trichloroethylidene glycol
28. Succinyl choline (Cl)	
29. Ergotamine (tartrate)	
30. Neostigmine (Br)	*m*-Hydroxyphenol trimethylammonium dimethylcarbamate
31. D-Tubocurarine ($2Cl \cdot {}_4H_2O$)	
H. Miscellaneous chemicals	
32. MS-222	Ethyl-*m*-aminobenzoate methane sulfonic acid

33. Iodoacetic acid
34. Piperonal butoxide — 3,4-Methylenedioxy-6-propylbenzyl-*n*-butyl diethylene glycol ether
35. EDTA ($2Na \cdot 2H_2O$) — Ethylenediamine tetraacetic acid

I. Inorganic salts (active cation)

36. $AgNo_3$
37. $AlCl_3$
38. $CuCl_2 \cdot 2H_2O$
39. NaCl
40. $CoCl_2 \cdot 6H_2O$
41. $HgCl_2$
42. $ZnCl_2$
43. $CdCl_2 \cdot 2\frac{1}{2}H_2O$
44. $FeCl_2 \cdot 4H_2O$
45. $FeCl_3 \cdot 6H_2O$
46. $Pb(NO_3)_2$
47. $NiCl_2 \cdot 6H_2O$
48. $BaCl_2 \cdot 2H_2O$
49. $MgCl_2 \cdot 6H_2O$
50. $CaCl_2 \cdot 2H_2O$
51. $CrCl_3 \cdot 6H_2O$
52. $PdCl_2$
53. K_2PdCl_6
54. $BeCl_2$
55. NH_4Cl
56. H_2O_2
57. AuCl
58. $HAuCi_4 \cdot 3H_2O$
59. $H_2PtCl_6 \cdot 6H_2O$
60. $SnCl_2 \cdot 2H_2O$
61. $SnCl_4 \cdot 5H_2O$
62. SeO_2

J. Inorganic salts (active anion)

63. Na_2HAsO_4
64. $NaAsO_2$
65. KCN
66. NaF
67. NaN_3
68. $Na_2S \cdot 9H_2O$
69. $NaIO_4$
70. $NaBH_4$
71. $K_2Cr_2O_7$
72. $Na_2HPO_3 \cdot 5H_2O$
73. $NaNO_3$
74. $NaNO_2$

TABLE 2

MOLARITY OF TEST CHEMICAL IN REACTION MIXTURE CAUSING 50% REDUCTION OF ENZYME ACTIVITY (I_{50}) FOR AChE–CHEMICAL INTERACTION, IN DECREASING ORDER OF EFFECT

Chemical (active component)	I_{50}	Chemical (active component)	I_{50}
Eserine	3.2×10^{-7}	Caffeine	5.0×10^{-3}
Neostigmine	7.1×10^{-7}	Diazinon	5.0×10^{-3}
Carbaryl	1.0×10^{-5}	Fe^{3+}	5.7×10^{-3}
Malaoxon	1.8×10^{-5}	Phenyl Hg	5.7×10^{-3}
AsO_2^-	3.2×10^{-5}	Adrenaline	5.7×10^{-3}
Cu^{2+}	1.6×10^{-4}	Noradrenaline	5.7×10^{-3}
Au^{3+}	5.0×10^{-4}	Bufotenin	5.7×10^{-3}
Pd^{2+}	5.0×10^{-4}	Atropine	5.7×10^{-3}
Cd^{2+}	5.7×10^{-4}	Nicotine	5.7×10^{-3}
Pd^{4+}	6.4×10^{-4}	Malathion	5.7×10^{-3}
Ag^+	1.0×10^{-3}	Pb^{2+}	7.1×10^{-3}
Tubocurarine	1.0×10^{-3}	Cr^{3+}	7.1×10^{-3}
IO_4^-	1.0×10^{-3}	Co^{2+}	7.1×10^{-3}
Au^+	1.4×10^{-3}	IPC (C 4)	8.0×10^{-3}
Hg^{2+}	1.6×10^{-3}	Phenacetin	1.0×10^{-2}
Sn^{2+}	1.8×10^{-3}	Aldrin	1.0×10^{-2}
Pt^{4+}	2.2×10^{-3}	Zn^{2+}	1.0×10^{-2}
$Cr_2O_7^{2-}$	2.2×10^{-3}	Serotonin	1.0×10^{-2}
Sn^{4+}	2.2×10^{-3}	MS-222	1.3×10^{-2}
Dieldrin	2.2×10^{-3}	F^-	1.4×10^{-2}
Ergotamine	2.5×10^{-3}	Al^{3+}	1.8×10^{-2}
AsO_4^{3-}	3.5×10^{-3}	PAM	1.8×10^{-2}
Eptam	4.0×10^{-3}	Be^{2+}	3.2×10^{-2}
Thiram	4.5×10^{-3}	[a]	$< 1 \times 10^{-2}$
Methyl Hg	5.0×10^{-3}	[b]	0

[a] Slight effect at 10^{-2} M(I_{50} not determinable): PCMB, succinyl choline, muscarine, chloral hydrate, iodoacetic acid, piperonal butoxide, N_3^-, NO_2^-, NO_3^-.

[b] Negligible effect at 10^{-2} M(I_{50} not determinable): lindane, captan, DDT, EDTA, SeO_2, H_2O_2, Fe^{2+}, Ni^{2+}, Ca^{2+}, Mg^{2+}, Ba^{2+}, Na^+, Cl^-, CN^-, S^{2-}, BH_4^-, NH_4^+, [redacted], PO_3^{3-}.

metabolically biotransformed from thio- to oxy-analogues. In this study malathion was about 570 times less active than carbaryl. A great many chemicals, in addition to carbamates and organophosphates, cause physiological and behavioral changes indicating neurochemical impairment which may involve AChE (Myers, 1974; Paulson, 1977).

Among the inorganic chemicals arsenite ion was highly inhibiting to AChE, and significantly more so than the arsenate ion. The transition metal cations in general are very toxic to fish and other animals and are also inhibiting to this enzyme. The order of effect was: $Cu^{2+} > Au^{3+} = Pd^{2+} > Cd^{2+} > Pd^{4+} > Ag^{1+} > Au^{1+} > Hg^{2+} > Sn^{2+} > Pt^{4+} > Sn^{4+}$. Other inorganic cations had less effect, and the alkaline and alkaline–earth cations had no measurable effect, with the possible exception of Be^{2+}. With reference to eserine, which had an I_{50} value of 3.2×10^{-7} (M), all of the

reagents that were about 33000 times less active were arbitrarily considered to be nonreactive and nonspecific for this enzyme.

The oxidizing anions, periodate and dichromate, were vigorous inhibitors of the enzyme. The other anions studied did not strongly inhibit activity, although some of them, such as cyanide, are relatively toxic to animals. Intermediate effects were found for the thiomide captan; the thiocarbamates thiram and eptam; the carbamate IPC; the halogenated hydrocarbons aldrin, DDT, dieldrin, and lindane; the alkaloid nicotine; and the organophosphates malathion and diazinon. The organometallic water pollutants, methyl mercury and phenyl mercury, which are very toxic to fish and are often considered to be neurotoxins, were only mildly inhibiting to AChE.

Four cholinergic-acting neurochemical agents, atropine, muscarine, nicotine, and PAM (pairs of which are believed to counteract each other in mammals), had only a moderate (but similar) effect on AChE. Likewise, two catecholamines, adrenaline, noradrenaline, and bufotenin and serotonin, indoleamines, had only mild effects. The drug tubocurarine was only mildly active, but was more active than ergotamine, caffeine, phenacetin, MS-222 (an anesthetic for fish), succinyl choline, and chloral hydrate. The strong SH-binding agents PCMB and iodoacetic acid were essentially nonreactive. Piperonyl butoxide, which potentiates the effects of certain pesticides, was slightly reactive. No significant effect was apparent from the oxidizing agent H_2O_2, the reducing agent BH_4^-, or the chelating agent EDTA.

No consistent pattern of inhibition was apparent between the action of oxidized and reduced states of inorganic chemicals. Thus, the order of inhibiting effect was: $Au^{3+} > Au^{1+}$, $Pd^{2+} > Pd^{4+}$, $Sn^{2+} > Sn^{4+}$, $Cr^{6+} > Cr^{3+}$, $Fe^{3+} > Fe^{2+}$, N^{3+} (in NO_2^-) = N^{5+} (in NO_3^-). However, as previously mentioned, As^{3+} (in AsO_2^-) was significantly more inhibiting than As^{5+} (inAsO_4^{3-}). It is not implied that in all cases the ions here drawn in simple form are the exact representation present in the reaction solution.

Further studies are needed to correlate structure with activity for all anticholinesterase reagents before AChE measurements and monitoring can be fully meaningful in clinical and environmental applications. If certain carbamates such as IPC are thousands of times less reactive than eserine, and if the arsenite ion, a noncarbamate, is much more reactive than some carbamates, AChE analyses cannot be unrestrictedly used to monitor carbamates and organophosphates. Considering the neurotoxicity to animals of many different chemical types of water toxicants it seems likely that other neurobiochemicals are involved, such as choline acetylase, certain amine oxidases or amine transferases, neurohormones, and perhaps biochemicals at receptor sites.

The mixture of 10 inorganic toxicants and the mixture of 10 organic pesticides both had a similar effect upon AChE activity. The initial preparation which was based upon the threshold concentration for each component caused a 100% loss of activity. Full enzyme activity was found after the mixtures were diluted 10-fold. The third mixture of 20 toxicants also showed a 100% inhibition by the initial preparation. After a 15-fold dilution 100% activity was found. These data indicate that at least some types of toxicants have an accumulative effect when they are

together in contact with an enzyme. This study is supportive of other work on the subject of the interrelationships of stressor factors in fish (Brown, 1968; Brown and Dalton, 1970; Snieszko, 1974; Dieter and Ludke, 1975; Christensen and Tucker, 1976) and emphasizes the need for further work in a complex area.

To protect animals fully, including humans, we must understand the mechanisms of toxic action and be wary of suggesting lower limits of exposure that may later prove to be unsafe. Toxicogenesis by water pollutants should be understood from the first molecular change and biochemical malfunction, through the stage of slight disability leading to more serious systemic changes, to the stages showing gross physiological impairment and general debility, leading to the ultimate irreversible consequences.

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